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(54) Title : A PROCESS FOR THE PREPARATION OF AN IMMUNO MODULATOR FROM THE AYURVEDIC MEDICINAL PLANT, GULVEL (TINOSPORA SP.)

(57) Abstract Gulvel is extensively used in ayurveda as a single or polyherbal formulations. Several plants of genus Tinospora, belonging to the family Menispermaceae, such as T.Cordifolia, T.malabarica, etc. are known by this name. Gulvel is used in a plethora of disorders ranging from fevers to rhumatism. The patent describes a process for the preparation of an immuno-modulator from the above ~~plants~~ plants. The process involves extraction of plant material with polar solvents, precipitation, isolation and purification of the active principle responsible for its ability to modulate the immune system. It is a polysachharide with highly branched macromolecular structure. It is polyclonally mitogenic to B-cells, and augments antibody response as well as enhances T-cell responses to model antigens.

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THE PATENTS ACT. 1970

PROVISIONAL

Specification

SECTION 10

The following Specification describes the nature of this invention :—

Several plants of the genus *Tinospora* such as *T. cordifolia T. malabarica, T. crispa* are commonly known as gulvel. They are also known as guduchi or amrita. Gulvel is highly valued for its medicinal properties and is extensively used in ayurvedic preparations. It belongs to the family Menispermaceae and is distributed in India, Pakistan, Sri Lanka and Myanmar. Many ayurvedic general tonics, anti-pyretic and diuretic preparations contain gulvel as one of the constituents. It has been claimed that gulvel is useful for the treatment of jaundice, skin diseases, diabetes, infections *etc*. Recently experimental evidences have been obtained which show that the extracts from *T. cordifolia* provide protection to animals against infection and sepsis under immunosuppressed conditions. Anti-phlogistic properties of gulvel have also been substantiated by experiments. Oral administration of aqueous extracts of gulvel has been reported to enhance the phagocytic activities of polymorphs.

Plants belonging to *Tinospora* species have been subjected to considerable phytochemical investigations. Isolation and characterization of diterpenoids, alkaloids, sterols, alcohols glucosides and phenolics have been reported. However, no attempts were made to relate the bioactivities to the chemical constituents. Our investigations on this medicinal plant have led to the development of a process for the isolation of an immunomodulatory active principle from gulvel. The immune system gets adversely affected under several conditions such as radiation exposure, cancer, surgery, organ transplantation and the body becomes susceptible to opportunistic pathogenic infections. Immunomodulators, therefore, find variety of therapeutic uses such as in radiation protection, cancer treatment and post-operative patient care.

The process involves extraction of the plant materials (all the parts) with solvents of different polarities. Individual extracts were subjected to bio-assay directed fractionation. Evaluation of mitotic activity provided a convenient bioassay to monitor the progress of fractionations. The bioassay consisted of culturing the spleen cells in the presence and absence of test samples and estimating the uptake of thymidine. The spleen cells, after removal of RBCs were cultured in a tissue culture medium containing controlled processed serum replacement(CPSR-2) in the presence of various amounts of extracts/isolated materials (aqueous solution) asceptically for 48 hr at 37°C in 5% CO_2 atmosphere. ^{3}H-Thymidine was added at the end of 48 hr incubation and 16 hr later the cultures were harvested on glass fibre papers and ^{3}H-Thymidine incorporation was quantitated by liquid scintillation counting. The thymidine uptake provided an index of mitotic activity. The plant materials were extracted with hexane, ether, chloroform, ethyl acetate, alcohols such as butanol, amyl alcohol, hot water and aqueous

alkali. Non-polar fractions did not show any mitotic activity. Diterpene glycosides and phenyl propanoids which were isolated from the alcoholic extract, showed only low mitotic activity while the aqueous extracts exhibited much higher activities. Therefore, efforts were directed towards isolation of the active principles from the aqueous extracts.

Increasing volumes of acetone (1-9 volumes) were gradually added to the vigouresly stirred aqueous extract of gulvel and precipitates were obtained. The precipitates were separated by filteration or centrifugation. Most of the bioactivity was located in the precipitates. The precipitates were re-dissolved in minimum volume of water and trichloroacetic acid (5-10% aqueous) was added. A precipitate, possibly of proteins, was obtained and separated by centrifugation. The supernant was diluted with excess of acetone and the precipitate thus obtained was separated by filteration/centrifugation. The precipitate was subjected to dialysis (with 10,000 Da cut off) to remove the low molecular weight compounds. Dialysed residue (Gl-1) was lyophilised to an amorphous fluffy powder which contained most of the mitogenic activity. Further purification was carried out by extensive gel permeation column chromatography using different gels such as Sephadex G-75, G-100 and G-200. The active fractions were further subjected to column chromatography on Sephacryl S-400. One hundred and fifty fractions (5ml each) were collected. Fractions Nos. 15-67 which contained very significant activity were combined. On rechromatography on different gels, Gl-1 was further fractionated into Gl-1A, Gl-2A, Gl-3A and Gl-4A. Gl-4 showed the highest mitogenic activity. Gl-4A was further purified by preparative high performance GPC (HP-GPC). Gl-4A is a white amorphous solid, moderately soluble in water but insoluble in chloroform, hydrocarbons, acetone and methanol. It gave positive Molisch's test Combustion of Gl-4A leaves an ash (4-6%). Elemental analyses of Gl-4A show the absence of nitrogen or sulphur. Complete acid hydrolysis (hydrochloric acid or trifluoro acetic acid) of Gl-4A gave a number of monosachharides which were analysed by paper chromatography and thin layer chromatography. Their identitites as rhamnose, galactose and arabinose, were established by the gas-chromatography-mass spectrometric (GC-MS) analysis of their alditol acetates [prepared by reduction with sodium borohydride followed by acetylation with acetic anhydride and pyridine]. Presence of substantial quantity galacturonic acid was inferred by its estimation by carbazole method. Methylation of Gl-4A by Hakamori's method (NaH, DMSO, CH_3I) gave a methyl ether which was hydrolysed with acid (hydrocloric acid or trifluoroacetic acid) to partially methylated monosachharides. The identitites of the partially methylated

monosachharides were established by the preparation of their alditol acetates followed by GC-MS analysis. The pattern of methylation revealed that Gl-4A is a highly branched polysachharide with several chains. Arabinose, galactose and rhamnose are the main terminal sugars. Most of the arabinose, galactose and galacuronic acid are present as 1-4 linked chain. Evidences for the cross-linkings between the chains through arabinose and galactose have also been obtained by the identification of appropriate partial methyl ethers. The inferences thus drawn were confirmed by enzymatic hydrolysis using several enzymes such as pectinase, α-galactosidase, α-glucosidase, β-glucosidase etc.

Extracts from gulvel and the active principle Gl-4A were found to be mitogenic to marine splenic lymphocytes. Analysis of the target sub population revealed that the extract as well as acetone precipitates and their fractions were polyclonally mitogenic to B cells only. They also enhanced the production of immunoglobulins (Ig) *in vitro* after culturing the cells with them for 5 days. The Ig secretion was quantitated by ELISA (enzyme linked immunosorbent assay). The extracts and purified preparations also enhanced antibody reponse of mice to sheep erythrocytes as assessed by Jerne's plaque forming cell assay (Cunningham modification), thus clearly establishing its immunomodulatory nature..

Dated the 24th October, the day of Friday 1997

For BHABHA ATOMIC RESEARCH CENTRE

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THE PATENTS ACT, 1970

COMPLETE

SPECIFICATION

SECTION 10

The following Specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed :-

This invention relates to a process for the preparation of a class of polysachharide from the ayurvedic drug gulvel having ability to activate cells of the immune system, enhance immune response, enhance the ability to fight infection and help overcome state of hypoactivity of the immune system as may be induced following chemotherapy or radiotherapy. Gulvel is extensively used in ayurveda as a single or in polyherbal formulations. Several plants of the genus, *Tinospora* belonging to family Menispermaceae such as *T. cordifolia, T. malabarica, T. crispa* and *T. sinensis* are known by the above mentioned name. They have wide occurrence in India. Gulvel is known by several names in different parts of India such as gulancha or padma gulancha in Bengali, ambavel or gulavel or giroli in Marathi and Gujarati amrita or amritolata or guduchi in Sanskrit, gulochi in Oriya, amrytu in Malayalam or amudom in Tamil. In ayurveda, it is classified as "Rasayana" and is used in plethora of disorders ranging from fevers to rheumatism. Bitter principle present in the drug show antispasmotic, antipyretic and antiinflammatory properties. The aqueous extract of stem known as giloe-ka-satva is used as a tonic. Their decoction is given in gout. Root is a powerful emetic and is also used for treatment of leprosy. Pulverized fruit is used for treatment of jaundice and rheumatism.

Gulvel offers many challenges to modern pharmacologists and phytochemists. Claims about gulvel in ayurveda were confirmed by different laboratories by experimenting on animal models and then extending to the clinical trials. The anti - inflammatory, analgesic and antipyretic effects of *T. cordifolia* aqueous extract were due to its diuretic efficacy and increase in nor-adrenaline levels. The stems of the plant have been used for kidney ailments. Its was found that *T. crispa* extract improved diabetic conditions in mice. The biochemical evidence for hypoglycaemic activity when orally administered and insulinotropic activity on human and rat islets has been reported. Aqueous extracts of *T. cordifolia* have been reported to enhance the defence mechanism of the host against infections.

It has been shown that oral administration of the aqueous extract protected rats against intestinal sepsis and mice against *E. coli* peritonitis. In mice given oral dose of the aqueous extract of *T. cordifolia* stems enhanced phagocytic activity in polymorphs and that appeared to prevent death due to abdominal sepsis. Even lowering of histamine levels following treatment with *T. cordifolia* has been reported. Such extracts modified the immunosuppression in rats infected with obstructive jaundice. Prior administration of these extracts significantly prevented myelosuppression induced by the cytotoxic drug cyclophosphamide. This effect, at least, partly could be attributed to enhanced leucocytosis evinced from the increase in total leucocyte and polymorphonuclear cell counts. Recently, the antioxygenic, antiviral, anti-AIDS and antineoplastic activity of gulvel extracts have been reported. The extracts of gulvel are also known to have anti-stress adaptogenic properties.

In view of oral efficacy and relative lack of adverse effects, Gulvel is an important drug with multifarous bioactivities. Although enough evidences have been collected for the biological activity, reports on isolation and characterisation of the active principles are scanty. The present invention deals with the isolation of the main active principles responsible for its ability to modulate the immunolgical capability of experimental animals such as induction of proliferation and differentiation in lymphocytes, secretion of antibodies on immunisation with model antigens and amelioration of the immunosuppressed state and related activities.

Numerous classes of compounds have been isolated from the plants belonging to *Tinospora* sp. They include diterpenoids such as clerodanes and norclerodanes, phenolic lignans, phenyl propanonoids, sterols, aliphatic alcohols, polysachharides, quartenary alkaloids, phytoecdysoids, flavonoids, *etc.* However, not many serious attempts have been reported to corelate the bioactivity with chemical constitutents of the plants.

Our research work has led to the isolation and characterisation of an active principle of Gulvel. This invention relates to a process for the isolation of this immunomodulatory active principle. It is essentially a mixture of closely related polysachharides with high molecular weights (1-5 x 10^6 D). The isolate shows high mitogenic activity to mouse B-lymphocytes and enhanced their immune response. The process of invention may be carried out by sequential partitioning the polar extracts with solvents of increasing order of polarities. Alternately, the isolation can be carried out by extracting the plant materials sequentially with non-polar to polar solvents. The fractionations were guided by a convenient bioassay related to evaluation of mitotic activity. The bioassay consists of culturing the mouse spleen cells in presence and absence of test samples and estimating the uptake of ^3H thymidine. After removing RBC's from the spleen cells, they are cultured in a tissue culture medium containing controlled processed serum replacement (CPSR-2) in presence of various amounts of extracted and isolated materials for 48 hrs. at 37°C in 5% CO_2 atmosphere. Tritiated thymidine is added at the end of 48 hrs. After incubation for 16 hrs, the cultures are harvested on glass fibre papers. The incorporation of tritiated thymidine is quantified by liquid scintillation counting. The thymidine uptake provides an index for mitotic activity. Non polar fractions do not show any activity. As the activity is exhibited by polar fractions, phytochemical investigation of polar extract is undertaken.

Fractions showing bioactivities were combined and isolation of the crude product was carried out by selective precipitation using chemicals such as alcohols or ketones. The crude products were purified by extensive chromatographic techniques such as adsorption chromatograpy, gel permeation chromatography, high performance gel permeation chromatography. The product was characterised by determination of the molecular size, elemental analysis, chemical and enzymatic hydrolysis. Further characterisation was done by chemical and physical techniques such as permethylation, acetylation, partial hydrolysis, high field NMR spectroscopy, mass spectroscopy *etc.*

The invention is described in greater detail in the following examples:

1.0 EXTRACTION PROCEDURE

Example 1.1

The mature plant of gulvel was collected during March-April. The stem of the plants was cleaned, dried in shade for several days. It may also be dried in oven at 50°C for several hours. After drying it was ground to a coarse powder using a blender. The dried material was extracted with various solvents such as different fractions of petroleum ether (40°-60° ;60°-80°), heptane, ether, chloroform, ethyl acetate, methanol, acetone, water, aqueous methanol and aqueous 1% alkali solution. The cold extraction is carried out in a percolator by packing the material lightly in it and then soaking it in the required solvent. The filtrate is collected after every 2 days. This procedure is repeated (3 times) with fresh solvents every time. Various extracts obtained are concentrated using rotavap or under vacuum at 40°C. Each extracts are assayed for mitotic activity.

Example 1.2

The fresh stem of matured plant, *Tinospora malabarica* was extracted with ethanol or methanol by percolation. The extraction was carried out for 6 days. It was repeated three times. The most of the solvent was removed by distillation under reduced pressure (water pump). The aqueous residue was extracted successively with petroleum ether (60°-80°) followed by diethyl ether, ethyl acetate and butanol. Crude extracts were obtained by the removal of the respective solvents by distillation under reduced pressure. The aqueous part left after solvent extraction was concentrated by evaporation. The crude concentrates were subjected to bioassay for evaluation of their mitotic activity.

Example 1.3

Using the procedure described in example 1.2, the stem, roots and leaves of *T. cordifolia* were extracted using solvents mentioned in the example. The crude extract was subjected to bioassay.

Example 1.4

The extraction of gulvel was carried out in boiling solvents such as heptane, diisopropyl ether, ethyl acetate methyl butyl ketone in a soxhlet extractor. The solvents were removed by distillation. Crude fractions were evaluated for bioactivity.

Example 1.5

The stem of *T. cordifolia* was cut into small pieces and crushed in a pestle and mortar. The crushed material was charged into a percolator and 2 volumes of 50% aqueous methanol or ethanol was added. After leaving the crushed material for 5 days, the extract was collected and reduced to half the original volume by distillation. The extraction was repeated 3 times. The combined concentrate was partitioned with equal volumes of chloroform (3 times), ethyl acetate (3 times), amyl acetate (2 times). Solvents were removed by flash evaporation to obtain the crude extracts.

Example 1.6

Four volumes of water was added to the crushed gulvel placed in a round-bottom flask and heated on steam bath for 10 hours. Water extract was removed by decantation and the extraction was repeated four to six times. The combined water extract was concentrated to one-third of its volume by flash evaporation. The concentrate was tested for bioactivity.

Example 1.7

The extraction was carried out as described in example 1.6 except that it was carried out a room temperature.

Example 1.8

Following the procedure described in example 1.6, extraction was carried out using 1% aqueous alkali. The extract was partitioned with solvents as described in example 1.2.

2.0 PURIFICATION OF THE EXTRACTS

The immunomodulatory activities were distributed in the polar extracts. For further purifications, the extracts were subjected to fractionation using chemical separation, selective precipitation, column chromatography, gel permeation chromatography and high

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Example 1.7

The extraction was carried out as described in example 1.6 except that it was carried out a room temperature.

Example 1.8

Following the procedure described in example 1.6, extraction was carried out using 1% aqueous alkali. The extract was partitioned with solvents as described in example 1.2.

2.0 PURIFICATION OF THE EXTRACTS

The immunomodulatory activities were distributed in the polar extracts. For further purifications, the extracts were subjected to fractionation using chemical separation, selective precipitation, column chromatography, gel permeation chromatography and high

performation gel premeation chromatography. The details are described in the following examples.

Example2.1

The polar extracts obtained as described in example 1.6 to 1.8 were lyophilised. The residue was taken up in minimum volume of water and filtered by passing through muslin cloth. To the filtrates, equal volume of ethanol, acetone or methanol were added. The precipitate thus formed was centrifuged. To the supernant another volume of methanol was added and the precipitate were removed by centrifugation. This was repeated till no precipitates were obtained (3-4 times).

Example 2.2

The precipitates obtained in the example 2.1 were put into dialytic cellulose tube with molecular weight cut off 5000 to 10,000. Both the low and high molecular weight fractions were evaluated for bioactivity. Most of the activity was found in the high molecular weight fraction.

Example 2.3

The precipitate obtained by the procedure described in example 2.2 was taken up in water and filtered. Equal volume of 10-15% aqueous trichloroacetic acid (TCA) solution was added. The precipitate thus obtained was filtered. To the filtrate, an equal volume of acetone or ethanol was added. The resulting precipitate was filtered. The residue was dissolved in minimum amount of water and dialysed to remove low molecular weight components. The activity was found in the high molecular weight fraction, which was lyophilised.

Example 2.4

The lyophilised solid was put for gel permeation chromatography using different sephadex such as G-75, G-100, G-200. Water was used for elution, 120 fractions (5ml each) were collected. The individual fractions were lyophilised. The fractions were analysed by high performance chromatography using phenomenex (Type polysep GFC-D 5000 No. 1427706) column. The molecular weights range was estimated by HPGPC based on

comparison with the elution behaviour of standard dextran markers. The molecular weight was in range of 1-5 x 10^6 D.

3.0 Characterisation

The active principle is obtained as white a fibrous solid. It dissolves in water in small quantities but at higher concentrations it forms a gel. It is insoluble in usual organic solvents such as methanol, chloroform, ether, benzene etc. It gives pink colour with Molisch's test, phenol-sulfuric acid and carbazole test. On heating it leaves small amount of ash (4-8%). It is optically active but specific rotation could not be determined with accuracy due to limited solubility in usual solvents. The solid obtained as described in example 8 was dissolved in water and subjected to hydrolysis by 2N hydrochloric acid for 6 hours on a steam bath. The hydrolysate was neutralised with 1N sodium hydroxide and dried by lyophilization. Paper chromatography of the hydrolysate revealed the presence of glucose, galactose, rhamnose, arabinose, xylose and galacturonic acid as the main monosachharides. The analysis was confirmed by thin-layer chromatography. The quantitative analysis was carried by converting the hydrolysate to alditol acetate and estimating the individual monosachharides by gas liquid chromatography (GLC).

Further information on structure was obtained by additional experiments. The active principle was methylated by Hakimori's method (DMSO, NaH, CH_3I). The per methylated product was hydrolysed by trifluroacetic acid, reduced with sodium borohydride and acetylated when the corresponding alditol acetates were obtained. The analysis of the hydrolysate was done by GC-MS. The presence of galacturonic acid was inferred by the positive (pink) carbazole test. Percentage of galacturonic acid was determined by quantitative carbazole assay. Analysis of the partially methylated active principle showed that most of the galactose, galacturonic acid and arabinose are present as 1-4 linked sugars. Evidence for the presence of arabinose, galactose and rhamnose as terminal sugars was also obtained. Identification of partially methylated sugars suggest cross linking amongst the linear chains through galactose and arabinose residues, which was characterised as arabinogalactan.

We claim:

1. A process for the preparation of an immunomodulator from the ayurvedic medicnal plant, Gulvel (*Tinospora sp.*) also known as amrita, guduchi, gulochi, giroli where

a) the dried & powdered or otherwise the whole plant material is sequentially extracted with solvents of increasing polarities such as hexane, diethyl ether, chloroform, ethyl acetate, methanol or ethanol, water and aqueous alkali;
b) the active principle (which was characterised as a branched arabinogalactan- a polysaccharide having a molecular weight in the range of $1\text{-}5\times10^6$ D) is selectively precipitated from the polar extracts obtained above in water medium by alcohols or ketones, preferably methanol;
c) the precipitate obtained above is dissolved in water and the inactive materials such as proteins are removed by precipitation with tricholoroacetic acid (5-20% by volume) and the enriched bioactive polysaccharide is again precipitated by the addition of alcohols or ketones;
d) the low molecular weight inactive compounds are removed from the precipitate obtained above by dialysis;
e) further purification of the active polysaccharide is carried out by the repeated gel permeation coloumn chromatography and preparative high performance gel permeation chromatography.

2. A process for the preparation of an immunomodulator from the ayurvedic medicinal plant, Gulvel (*Tinospora sp.*) substantially as described in the specifications with examples 1.1 to 1.8 and 2.1 to 2.4.

Dated this 20th day of October 1998.

for BHABHA ATOMIC RESEARCH CENTRE

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